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Physical Mapping of the Holoprosencephaly Critical Region in 21q22.3, Exclusion of *SIM2* as a Candidate Gene for Holoprosencephaly, and Mapping of *SIM2* to a Region of Chromosome 21 Important for Down Syndrome

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Summary

We set out to define the holoprosencephaly (HPE) critical region on chromosome 21 and also to determine whether there were human homologues of the *Drosophila single-minded* (*sim*) gene that might be involved in HPE. Analysis of somatic cell hybrid clones that contained rearranged chromosomes 21 from HPE patients defined the HPE minimal critical region in 21q22.3 as D21S113 to qter. We used established somatic cell hybrid mapping panels to map *SIM2* to chromosome 21 within subbands q22.2-q22.3. Analysis of the HPE patient–derived somatic cell hybrids showed that *SIM2* is not deleted in two of three patients and thus is not a likely candidate for *HPE1,* the HPE gene on chromosome 21. However, *SIM2* does map within the Down syndrome critical region and thus is a candidate gene that might contribute to the Down syndrome phenotype.

Introduction

Holoprosencephaly (HPE) is a common craniofacial anomaly that affects 1/16,000 newborn infants and has an extremely variable phenotype (Cohen 1989*a,* 1989*b;* Cohen and Sulik 1992). The spectrum of brain malformations ranges from a single brain ventricle, in the most severe form, to milder midline CNS defects resulting in absence or hypoplasia of the corpus callosum, olfactory bulbs and tracts, and/or optic bulbs and tracts. In ~80% of HPE patients, severe CNS anomalies are accompanied by characteristic facial features. The facial anomalies range in severity from cyclopia to more mild manifestations such as hypo- or hypertelorism, midline or lateral clefts of lip and/or palate, a single central incisor, and others. Clinically unaffected obligate carriers of autosomal dominant HPE with normal brains and faces have been reported (Muenke et al. 1994).

The etiology of HPE is heterogeneous: in some cases, there is evidence for the involvement of teratogens, and in others genetic factors are suggested by the observation of familial occurrence and nonrandom chromosomal anomalies in patients with HPE (Muenke 1994). Deletions or other cytogenetic rearrangements involving specific, nonrandom regions of chromosomes 2, 3, 7, 13, 18, and 21 have been reported to be associated with HPE (Muenke 1989; Gurrieri et al. 1993; Overhauser et al. 1995; U. Schell and Max Muenke, unpublished data). Three cases of HPE associated with unbalanced translocations or deletions resulting in partial monosomy of human chromosome 21 have been reported (Aronson et al. 1987; Ippel et al. 1991; Niedermeyer et al. 1994), suggesting that monosomy of a gene(s) designated *HPE1* (Frézal and Schinzel 1991) could be necessary, and perhaps sufficient, to cause HPE. Thus, the first step in understanding HPE associated with partial monosomy 21 was to define the regions deleted in such cases, particularly the smallest region of deletion overlap in common with all patients. A search for candidate genes could then be narrowed to this HPE minimal critical region.

A complementary approach to the molecular analysis of HPE is to identify candidate genes on the basis of known involvement in midline brain development, which might be involved in HPE (Collins 1995). In *Drosophila,* the *sim* gene is known to encode a basic helix-loop-helix (bHLH) protein, which is involved in CNS midline development (Crews et al. 1988, 1992; Nambu et al. 1990, 1991). In the mouse, two homologues of the *sim* gene have been identified, *Sim-1* and *Sim-2* (C.-M. Fan and M. Tessier-Lavigne, unpublished data).

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The expression of *Sim-2* in the CNS is in the forebrain/midbrain region (mouse embryonic day E8.5), the ventral-caudal portion of the diencephalon (E9.5), and the rostral and caudal portions of the hypothalamus, regions potentially involved in HPE. *Sim-1* has a lower level of expression in the forebrain/midbrain region (E8.5) than does *Sim-2* but is otherwise expressed in a pattern similar to that of *Sim-2* (C.-M. Fan and M. Tessier-Lavigne, unpublished data). Therefore, the human homologues of *Sim-1* and *Sim-2* could potentially be involved in HPE and warranted examination as candidate genes.

Patients, Material, and Methods

Human Cell Lines

Lymphoblastoid cell lines (LCLs) were established from HPE patients with deletions of various regions of chromosome 21 or from the parent carrying a balanced translocation involving chromosome 21. Informed consent was obtained in accordance with the standards set by local institutional review boards. LCL589, 46,XX, del(21)(q22.3) was derived from an HPE patient with a de novo deletion of part of 21q22.3 (Estabrooks et al. 1990). LCL632, 46,XY,t(11;21)(p15.5;q22.3), was derived from the father of a patient with HPE (Ippel et al. 1991). The affected individual (632P) inherited the unbalanced translocation karyotype, which resulted in deletion of 21(q22.3→qter) and duplication of 11(pter→p15.5). LCL697 (GM06135), 46,XX,t(10;21)(p11.2;q22.3), was derived from the mother of a patient (698, GM06136) with HPE microsigns and an unbalanced translocation resulting in a deletion of part of 21(q22.3→qter) and duplication of 10(pter→p11.2).

Somatic Cell Hybrid Production

Somatic cell hybrids carrying the derivative 21 chromosome, der(21), were produced by Sendai virus mediated–fusion of the human LCLs with $ADe^{-}C$ mutant Chinese hamster ovary cells as described elsewhere (Van Keuren et al. 1986). The gene encoding GART, which is mutated in $ADe^{-}C$ cells, has been localized to 21q22.1 and thus would be expected to be present in each of the der(21) chromosomes of interest and could serve as a selectable marker. Adherent cells containing the der(21) or del(21) chromosomes, which are able to grow in the purine-free medium, were isolated from each fusion and subjected to analysis as described below.

Cytogenetic Analysis of Somatic Cell Hybrids

Individual somatic cell hybrid clones were analyzed cytogenetically by standard Giemsa-trypsin-Giemsa (GTG) banding methods and by FISH, to metaphase chromosomes for the presence of the der(21) with a chromosome 21 paint probe, a chromosome 10 paint probe, a 21/13 centromere-specific probe (Oncor), a cosmid clone containing DNA marker D11S813E (H19), which was previously mapped to the chromosome 11p11→pter region (Glaser et al. 1989), and a cosmid containing chromosome 21-specific sequences very close to the chromosome 21qter telomere (Oncor), using previously published methods (Shechter et al. 1994). Probes were labeled with digoxigenin, and post-hybridization washes were done with 50% formamide/1 × SSC and 1 × SSC at 42°C for 15 and 8 min, respectively. Signal detection was done using a digoxigenin-FITC detection kit (Oncor). DNA cosmid probe D21S203EID, specific for the telomeric region of 21q22.3, was hybridized concurrently with an alpha-satellite DNA probe for chromosome 21 centromere (Oncor) on both the human lymphoblastoid cell line LCL589 and the hamster-human hybrid line 589C-4B (LCL589 × $Ade^{-}C$). The 21q22.3 cosmid probe was negative on the del(21) chromosome in both the patient's cell line and the hybrid cell line, indicating that the hybrid clone 589C-4B contained only the del(21) from the patient's cell line. The chromosome 21 centromere probe was included to identify positively chromosome 21. Total chromosome 21 paint probe (Coatasome 21, Oncor) was positive on only one chromosome in the hybrid line, showing that no normal 21 or occult pieces of chromosome 21 existed in the hybrid cells.

PCR Analysis of Somatic Cell Hybrids

PCR analysis of somatic cell hybrids was performed using PCR primers corresponding to markers D21S315, D21S327, D21S331, D21S326, D21S332, D21S65, D21S330, AML1, D21S393, D21S211, CBR, D21S333, D21S334, D21S267, D21S335, D21S336, D21S270, D21S337, D21S55, D21S233, D21S259, D21S341, D21S342, ERG, D21S338, ETS2, D21S220, D21S346, D21S168, D21S15, D21S23, D21S231, D21S349, D21S350, D21S352, D21S354, D21S53, D21S64, BCE1, D21S358, D21S359, D21S399, D21S113, D21S360, CBS, D21S361, CRYA, PFKL, D21S401, D21S112, D21S403, and S100β. Markers are listed from the centromere to the telomere (Chumakov et al. 1992*a*, 1992*b*; Shimizu et al. 1995; S. Graw and D. Patterson, unpublished results). All primer sequences were as entered in the Genome Database (GDB).

Mapping of SIM1 *and* SIM2

Mouse genomic fragments of *Sim-2* (1.2 Kb) and *Sim-1* (4.0 Kb), each containing the bHLH region, were separated from the pBluescript vector on low-melting-point agarose gels and prepared using random priming (Boehringer Mannheim) with ^{32}P-dCTP for use as probe in Southern blot analysis. For the initial chromosomal localization of the human *SIM1* and *SIM2* genes, a monochromosomal hybrid panel (National Institute of

Table 1

Clinical and Cytogenetic Findings in Three Patients with Holoprosencephaly and Distal 21q Deletions

Patient	Age	CNS Findings	Facial Findings	Cytogenetic Anomalies	References
589	2 years	HPE	Normal	del(21)(q22.3→qter) 46,XX,del(21)(q22.3→qter)	Estabrooks et al. 1990
632P	Fetus	HPE	HPE, CLP, MO	del(21)(q22.3→qter); dup(11)(p15.5→pter) 46,XX,−21,+der(21),t(11;21)(p15.5;q22.3)pat	Ippel et al. 1991
698 (GM06136)	2 years	MC, MR	CLP	del(21)(q22.3→qter); dup(10)(p11.2→pter) 46,XY,−21,+der(21),t(10;21)(p11.2;q22.3)mat	

NOTE.—CNS findings consisted of alobar HPE and/or microcephaly (MC), developmental delay, or mental retardation (MR). HPE facial features consisted of cleft lip and/or palate (CLP) and microphthalmia (MO).

General Medical Sciences human/rodent somatic cell hybrid mapping panel 2 from the Corriell Cell Repositories) was used. DNA digestion for the Southern blot analysis was with *Hin*dIII for the *Sim-1*-specific probe (resulting in a 4-kb human and a 10-kb hamster genomic fragment) and with *Eco*RI for the *Sim-2*-containing probe (yielding a 5.4-kb human and a 3.8-kb hamster fragment). Hybridization was done in 50% formamide hybridization solution at 42°C overnight, using standard protocols. High-resolution mapping of *SIM2* on chromosome 21 was performed using Southern analysis of *Eco*RI-digested DNA from the following subset of a somatic cell hybrid mapping panel described in detail elsewhere (Gardiner et al. 1988; Graw et al. 1988; Langer et al. 1990): $21q^+$, $8q^-$, 2 Furl, 10;21, R210, ACEM, JC6, 6918-8al, MRC2, 3X1S, 1X4, 3X2S, and 1X18.

Results

Case Reports

All three patients were selected for deletions involving the long arm of chromosome 21 and clinical findings consistent with the holoprosencephaly spectrum (table 1). Patient 589 has alobar HPE, minor dysmorphic facial features, and developmental delay (Estabrooks et al. 1990). Patient 632P had alobar HPE (fig. 1), microcephaly, microphthalmia with retinal dysplasia, hypotelorism, bilateral cleft lip and palate, and extracranial anomalies such as hyperlobulation of the lungs and medullar renal cysts. Patient 698 had microcephaly, developmental delay at age 2 years, bilateral cleft lip and palate, and skeletal anomalies such as absent patellae.

Definition of the HPE1 Critical Region

To define molecularly the chromosome 21 regions missing from the del(21) and der(21) chromosomes, we generated 3 hamster × human somatic cell hybrid clones and confirmed by cytogenetic and FISH analysis that they contained these rearranged chromosomes in the absence of the normal chromosomes 21. PCR analysis was carried out with a subset of the DNA markers that map to 21q22.3 and some that span the long arm of chromosome 21 (Chumakov et al. 1992*a*, 1992*b*). This analysis allowed precise definition of the regions of chromosome 21 missing in each chromosome (fig. 2). The

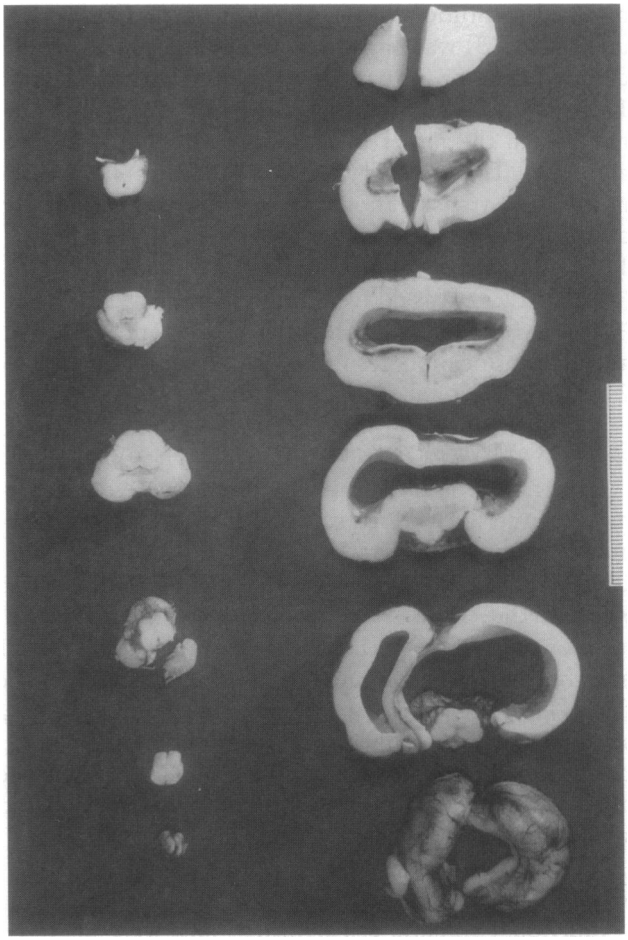

Figure 1 Alobar holoprosencephaly of the forebrain without any identifiable midline structures in patient 632P with bilateral cleft lip and palate and a small deletion of chromosome 21 as the result of a balanced translocation, t(11;21)(p15.5;q22.3)pat.

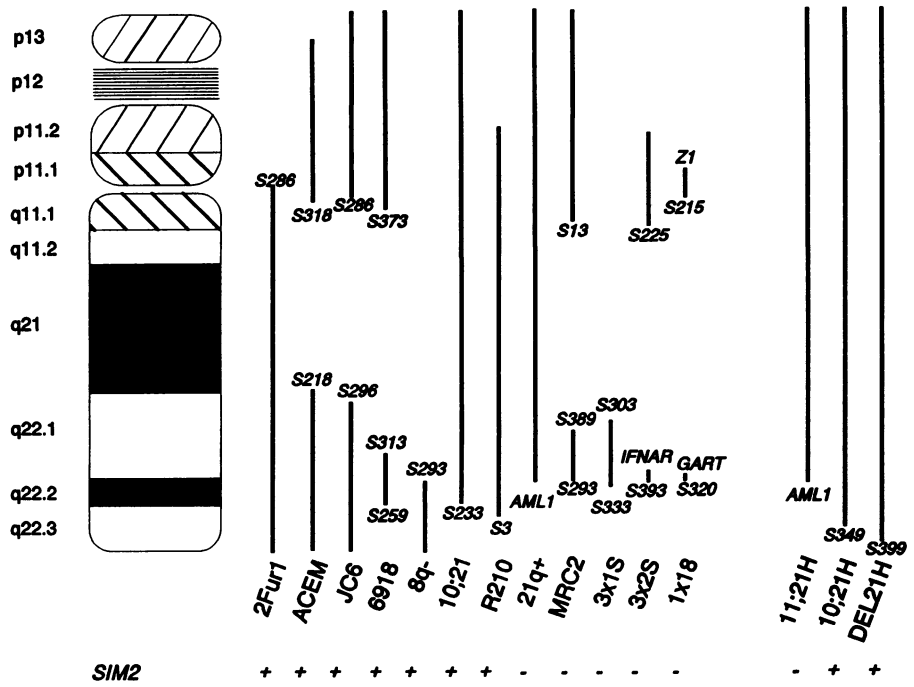

Figure 2 Schematic diagram of human chromosome 21. Vertical bars to the right of chromosome 21 represent somatic cell hybrids from our reference panel (first 12 bars) and somatic cell hybrids derived from the three HPE cell lines (last three bars) that contain partial regions of chromosome 21. These regions are defined by the DNA loci or genes, which are present above or below the respective bars. The three somatic cell hybrids 11;21H, 10;21H, and DEL21H, which are derived from holoprosencephaly cell lines, define the *HPE1* minimal critical region in 21q22.3 as D21S113, which is distal to D21S399, to 21qter. *SIM2* regional mapping on chromosome 21 is indicated (on the bottom) by the presence (+) or absence (−) of SIM2-specific sequences below the respective somatic cell hybrids. For the order of DNA markers from chromosome 21 centromere to the telomere see the Patients, Material, and Methods section.

der(21)t(11;21) chromosome is deleted for a region from 21qter (S100β) through D21S211, the der(21)t(10;21) chromosome is missing from qter (S100β) through D21S350, and the del(21) is missing from 21qter through D21S113. Thus, the minimal critical region of deletion overlap in all three HPE cell lines is within 21q22.3 between D21S113 and 21qter.

Mapping of SIM1 *and* SIM2

A human homologue of the *sim* gene presents an interesting candidate gene for HPE, since *sim* in *Drosophila,* and *Sim-1* and *Sim-2* in mouse, may be involved in midline brain development. Our initial experiments indicated that *SIM2* mapped to human chromosome 21 and that *SIM1* mapped to human chromosome 6. Since no association of chromosome 6 with holoprosencephaly has been reported, further regional localization of *SIM1* was not done. Southern blot hybridization analysis of a high-resolution chromosome 21–specific somatic cell hybrid mapping panel was performed to map the *SIM2* gene to 21q22.2-q22.3 between D21S333 and D21S341 (fig. 2). This mapping indicates that *SIM2* should be deleted in the t(11;21) chromosome but present in the t(10;21) and del(21) chromosomes. Southern analysis of the three somatic cell hybrids described earlier indeed indicates that *SIM2* is present in the der(21)t(10;21) and the del(21) containing hybrid clones but is deleted in the der(21)t(11;21) hybrid (fig. 2).

Discussion

We have defined chromosomal breakpoints on three new rearrangements of chromosome 21 and the region of chromosome 21 that is associated with HPE. This region, which contains the putative holoprosencephaly gene, *HPE1,* is located in 21q22.3 between D21S113 and 21qter and is <4.9 but ≥ 4.5 Mbp in size. Using a high-resolution somatic cell hybrid mapping panel, we mapped the *SIM2* gene to chromosome 21q22.2-q22.3 between D21S333 and D21S341. Most significantly, we have demonstrated that *SIM2* is not located in the region that is deleted in two of the three HPE-associated chromosome 21 regions defined here. Thus, we have excluded the human *SIM2* gene as a likely *HPE1* candidate gene.

Since the chromosome 21 physical map is so well defined, an accurate estimate of the size of the missing regions of the der(21) chromosomes can be determined in Mbp of DNA. Using the provisional *Not*I contiguous map of chromosome 21 agreed on at the Fourth Interna-

tional Workshop on Human Chromosome 21 (Delabar et al. 1993), the following estimates can be made. The der(21)t(11;21) deletion includes ~12.4 Mbp from 21q22.3, the der(21)t(10;21) deletion no more than 7.3 Mbp, and the del(21) no more than 4.9 Mbp. In addition, analysis of markers from BCE1 to S100β suggests that the deletion in the del(21) chromosome must be ≥4.5 Mbp, extending from the telomere through D21S113. These results define the minimal deleted region associated with HPE as a 4.5–4.9-Mbp region between D21S113 and 21qter. This region contains several known genes, including S100β, COL6A1, COL6A2, CD18, CRYA, CBS, and PFK6, that could be considered as candidate genes.

These three new breakpoints will increase the resolution of the chromosome 21–specific somatic cell hybrid mapping panel in this gene-rich 21q22.3 region of the chromosome. This is likely to be particularly important in generating an integrated high-resolution map of chromosome 21, since this region of the chromosome is particularly difficult to cover with stable, unrearranged YACs (Chumakov et al. 1992*a*). By narrowing the region in which the putative *HPE1* gene is located, additional candidate genes can be identified and studied for their possible role in holoprosencephaly.

SIM2 maps to 21q22.2-q22.3 and is not deleted in two of three HPE patient derived somatic cell hybrids. This provides evidence that *SIM2* is not a candidate for the *HPE1* gene, since the distance between the distal ends of the der(21) chromosomes is too large to account for breakage within *SIM2* in both cases.

While these studies rule out *SIM2* as a candidate gene for *HPE1*, the regional mapping of this gene on chromosome 21 does indicate that *SIM2* should be considered as possibly playing a role in the developmental anomalies seen in individuals with Down syndrome. Indeed, *SIM2* appears to lie within the region commonly referred to as the "Down syndrome critical region" (DSCR). The DSCR has been defined by several investigators and may extend from D21S17 to *ETS2* (Antonarakis et al. 1995) and virtually always contains D21S55 (Peterson et al. 1994). While the exact interpretation of these studies is complex (Korenberg et al. 1994), clearly this region of chromosome 21 contains genes that make a significant contribution to the phenotype of Down syndrome. D21S17 and *ETS2* flank the region within which we have mapped the *SIM2* gene, and D21S55 is within this region. On the basis of this location, *SIM2* is clearly a good candidate to be a gene responsible for some of the Down syndrome phenotype. In *Drosophila*, the *Sim* gene encodes a transcription factor that acts as a master regulator for CNS development (Nambu et al. 1991). If the human *SIM2* gene acts similarly, this would also emphasize that it should be examined in detail as a candidate gene responsible for some features in Down syndrome.

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